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No single way to understand singlet oxygen signalling in plants

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When plant cells are under environmental stress, several chemically distinct reactive oxygen species (ROS) are generated simultaneously in various intracellular compartments and these can cause oxidative damage or act as signals. The conditional *flu* mutant of *Arabidopsis*, which generates singlet oxygen in plastids during a dark-to-light transition, has allowed the biological activity of singlet oxygen to be determined, and the criteria to distinguish between cytotoxicity and signalling of this particular ROS to be defined. The genetic basis of singlet-oxygen-mediated signalling has been revealed by the mutation of two nuclear genes encoding the plastid proteins EXECUTER (EX)1 and EX2, which are sufficient to abrogate singlet-oxygen-dependent stress responses. Conversely, responses due to higher cytotoxic levels of singlet oxygen are not suppressed in the *ex1/ex2* background. Whether singlet oxygen levels lower than those that trigger genetically controlled cell death activate acclimation is now under investigation.

Keywords: *Arabidopsis*; oxidative stress; singlet oxygen; *flu* mutant; *executer*

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Introduction

Reactive oxygen species (ROS) are produced continuously as unavoidable by-products of aerobic metabolism (Halliwell, 2006). Oxygen in its triplet ground state is chemically inert but it can be converted to a ROS either by electron transfer—leading to, by stepwise reduction, the formation of the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$)—or energy transfer—which leads to the formation of singlet oxygen ($^1O_2$; Foote, 1968; Gollnick, 1968). During evolution, the biological activities of ROS seem to have undergone several modifications. The continuous release of highly reactive ROS has necessitated the development of ROS scavengers to minimize the cytotoxic effects of ROS in the cell (Apel & Hirt, 2004). At the same time, the detection of rapid changes in ROS concentrations that result from metabolic disturbances or external factors has been used by cells to activate stress-related responses and to readjust their homeostasis (Gechev *et al,* 2006). Finally, the discovery of genetically controlled ROS production by enzymes such as NADPH oxidases has assigned a surprisingly large diversity of biological activities to ROS. These activities affect a range of processes, including the defence reactions of plants and vertebrates against pathogens (Sagi & Fluhr, 2006), the regulation of cell expansion and development (Foreman *et al,* 2003; Gapper & Dolan, 2006), and plant–fungus mutualistic interactions (Takemoto *et al,* 2006; Tanaka *et al,* 2006).

In plants, chloroplasts and peroxisomes are the main sites of ROS production (Asada, 2006; Foyer & Noctor, 2003). The enhanced generation of ROS in these cellular compartments has been attributed to the disturbance of light-driven photosynthetic electron transport by various environmental factors that trigger stress responses (Niyogi, 1999). Under these environmental conditions, plants are exposed to light intensities that exceed their capacity to assimilate CO_2 and lead to the over-reduction of the electron-transport chain, and ultimately can result in the inhibition of photosynthesis. Plants use two strategies to protect their photosynthetic apparatus: first, the thermal dissipation of excess excitation energy in the photosystem II (PSII) antennae—known as non-photochemical quenching (Muller *et al,* 2001); and second, the transfer of electrons from PSII to alternative sinks, thereby sustaining partial oxidation of PSII acceptors and preventing photoinactivation of PSII—known as photochemical quenching. Photochemical quenching can be achieved in chloroplasts through, for example, the direct reduction of oxygen to $O_2^{\bullet-}$ by reduced electron-transport components associated with PSI (Asada, 1999; Rizhsky *et al,* 2003) and by reactions linked to the photorespiratory cycle that result in the enhanced production of H_2O_2 in peroxisomes (Kozaki & Takeba, 1996). $^1O_2$ is continuously produced by PSII through energy transfer from excited chlorophyll to oxygen (Gollnick, 1968; Krieger-Liszkay, 2005). The quenching of $^1O_2$ has been linked primarily to the turnover of the D1 protein of the PSII reaction centre (Telfer *et al,* 1994; Trebst, 2003), although tocopherols and carotenoids also participate in preserving PSII from photoinactivation (Havaux *et al,* 2005; Krieger-Liszkay & Trebst, 2006; Telfer, 2005).

Plant responses to singlet oxygen

One of the difficulties in studying the biological activity of a particular ROS originates from the fact that several chemically distinct ROS are generated simultaneously in cells under stress, therefore

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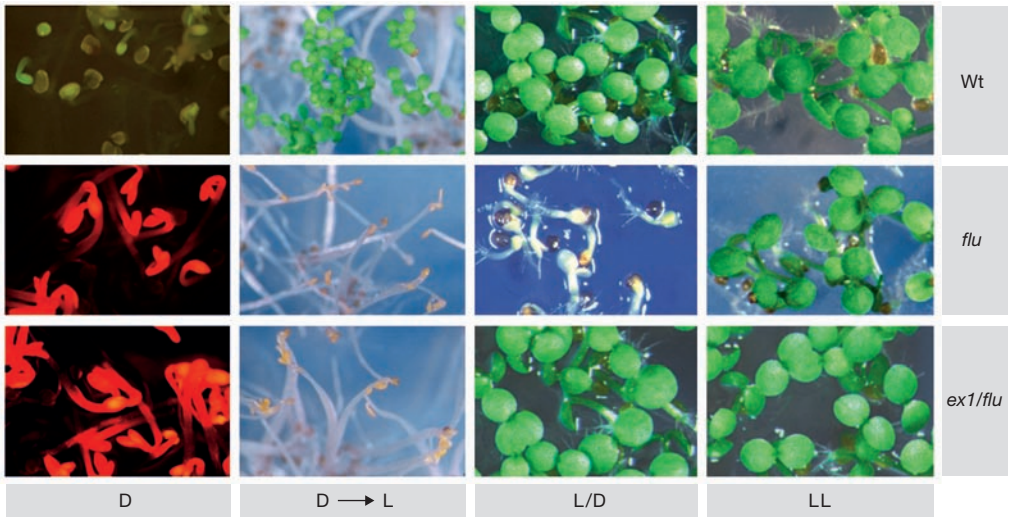

Fig 1 | Cytotoxicity versus signalling of $^1O_2$ in *Arabidopsis* seedlings. In etiolated *flu* and *executer1/flu (ex1/flu)* seedlings, similar excess amounts of free protochlorophyllide (Pchlide) accumulate that, on excitation with blue light, emit a strong red fluorescence (D). In pre-illuminated *flu* seedlings exposed to 16 h: 8 h light:dark cycles (L/D) inactivation of the EX1 protein abrogates bleaching. However, in etiolated *flu* seedlings, which accumulate four- to fivefold the amounts of free Pchlide in seedlings grown in continuous light and transferred to the dark for 8 h, the $^1O_2$-mediated collapse during re-illumination is not suppressed by the *ex1* mutation (D→L). All three plant lines grew equally well under continuous light (LL). Adapted from Przybyla *et al* (2008) and reprinted with permission from Wiley–Blackwell Publishing Ltd. Wt, wild type.

making it almost impossible to link a particular stress response to a specific ROS. However, to study the specific activity of $^1O_2$, this problem has been overcome by using the conditional *flu* mutant of *Arabidopsis,* which allows the induction of only $^1O_2$ within plastids in a non-invasive and controlled manner.

FLU is a nuclear-encoded protein that is tightly associated with plastid membranes. It regulates the Mg^{2+} branch of tetrapyrrole biosynthesis by interacting directly with glutamyl tRNA reductase (Meskauskiene *et al,* 2001; Meskauskiene & Apel, 2002). In contrast to the wild type, etiolated *flu* seedlings are no longer able to restrict the accumulation of protochlorophyllide (Pchlide)—the immediate precursor of chlorophyllide (Chlide)—which is a potent photosensitizer that generates $^1O_2$ in the light (Fig 1, D; Meskauskiene *et al,* 2001; op den Camp *et al,* 2003). As a result, when these seedlings are transferred from the dark to the light they rapidly bleach and die (Fig 1, D→L; Meskauskiene *et al,* 2001). However, the *flu* mutant remains viable when it is grown from germination under continuous light (Fig 1, LL). Under these conditions, Pchlide is immediately photoreduced to Chlide and therefore does not accumulate. Provided the *flu* plants are kept under continuous light, no obvious differences are observed between them and the wild type. This conditional phenotype of the *flu* mutant has been exploited to study the physiological role of $^1O_2$ by growing *flu* plants initially under continuous light—until they reach the developmental stage of interest—then transferring them to the dark and re-exposing them to light. The release of $^1O_2$ in the *flu* mutant has been determined directly by using the $^1O_2$-specific probe DanePy (Kalai *et al,* 1998; op den Camp *et al,* 2003). Seedlings of *flu* bleach and die when they are kept under repeated 16 h:8 h light:dark cycles (Fig 1, L/D), whereas mature plants grown initially under continuous light develop necrotic lesions on their leaves and stop their growth immediately after being moved to daily dark:light cycles (op den Camp *et al,* 2003).

These stress responses could result either from physicochemical damage caused by $^1O_2$ or by the activation of a genetically determined stress-response programme. Initially, we addressed this question by using experiments in which plants were grown under continuous light and then moved to one 8 h dark period before re-exposure to light.

Singlet-oxygen-responsive gene network

Under these experimental conditions, several global gene-expression studies have been carried out with *flu* plants using Affymetrix GeneChip arrays (Laloi *et al,* 2006, 2007; Lee *et al,* 2007; op den Camp *et al,* 2003). Within 30 min of the release of $^1O_2$ in plastids, approximately 300 nuclear genes are rapidly upregulated at least threefold in *flu* plants relative to the wild type. On the basis of comparative transcriptome analyses, we identified groups of genes that have been shown previously to also be under the control of phytohormones, in particular, abscisic acid, ethylene, methyl jasmonate and salicylic acid (Danon *et al,* 2005; Ochsenbein *et al,* 2006). Previous attempts to modulate the triggering of stress responses in *flu* plants by using phytohormone-related mutants revealed that the ethylene, methyl jasmonate and salicylic acid pathways do have a role in mediating the cell-death response of *flu* (Danon *et al,* 2005; Ochsenbein *et al,* 2006). Furthermore, we identified genes that seem to be more strictly under the control of $^1O_2$ (Gadjev *et al,* 2006; Laloi *et al,* 2006; op den Camp *et al,* 2003). In addition, approximately 50 genes encoding putative transcription factors showed a threefold increase in expression within 30 min of the release of $^1O_2$. These include ethylene responsive factors, WRKY transcription factors, zinc-finger proteins and several DNA-binding proteins. Many genes involved in putative signal-transduction pathways and calcium

regulation, such as protein kinases, calcium and calmodulin-binding proteins, were also identified (Danon *et al,* 2005; Laloi *et al,* 2006; Lee *et al,* 2007; op den Camp *et al,* 2003). We are now using genetic screens to identify modulators and constituents of the $^1O_2$ signalling network. We have searched for $^1O_2$-responsive and $^1O_2$-specific promoters that, in combination with the luciferase (LUC) reporter gene, can be used in the *flu* background to monitor $^1O_2$-dependent activation of nuclear genes. A *LUC* reporter gene line has been mutated with ethyl methane sulphonate (EMS), and second-site mutants with a constitutively upregulated *LUC* expression have been isolated. The preliminary characterization of these mutants supports our view that $^1O_2$ signalling does not operate as an isolated linear pathway, but rather merges in a complex signalling network that integrates various cues and relays these to the nucleus (A. Baruah, K. Simkova, C.L. & K.A., unpublished data).

The finding that $^1O_2$ can selectively activate nuclear genes, which are not—or only poorly—responsive to $O_2^{\bullet-}$ or H_2O_2, has also been reported for mammals (Klotz *et al,* 2003), the unicellular algae *Chlamydomonas* (Leisinger *et al,* 2001) and the phototrophic bacterium *Rhodobacter sphaeroides* (Anthony *et al,* 2005). In *Chlamydomonas*, a glutathione peroxidase homologous gene (*GPXH*) was shown to be strongly induced by $^1O_2$-generating photosensitizers, but only weakly induced by $O_2^{\bullet-}$ or H_2O_2 (Fischer *et al,* 2005; Leisinger *et al,* 2001). A second gene, encoding heat-shock protein (HSP)70A, was shown to be induced by both $^1O_2$ and H_2O_2. The study of its promoter has revealed that activation by $^1O_2$ might require promoter elements that are different from those used by H_2O_2 (Shao *et al,* 2007). In *Rhodobacter sphaeroides*, the group IV sigma factor sigma(E) is required to activate a specific transcriptional response to $^1O_2$, thereby protecting cells against this ROS (Anthony *et al,* 2005; Campbell *et al,* 2007; Glaeser & Klug, 2005). Protein synthesis patterns were also distinct from those after H_2O_2 treatment, but similar to those after high light exposure (Glaeser *et al,* 2007). Although the response to $^1O_2$ is regulated primarily by sigma(E), mutant strain analysis revealed the existence of sigma(E)-independent pathways (Glaeser *et al,* 2007). In addition, the *phrA* gene, which encodes a photolyase, is regulated by both $^1O_2$ and H_2O_2 in a sigma(E)-dependent manner, but not by treatment with paraquat, which generates $O_2^{\bullet-}$ that subsequently dismutates to H_2O_2 (Hendrischk *et al,* 2007).

Interactions of $^1O_2$ signalling with other ROS

These results strongly suggest that, in various photosynthetic organisms, $^1O_2$ and other ROS affect nuclear gene expression through distinct signalling pathways. These signalling pathways do not always operate independently and might interact with each other. In *Arabidopsis*, evidence supporting such interactions has been provided by non-invasively modulating the concentration of H_2O_2 in transgenic mutant plants that overexpress the plastid-specific thylakoid-bound ascorbate peroxidase (Murgia *et al,* 2004). The overexpression of this H_2O_2 scavenger enhanced the intensity of $^1O_2$-mediated stress responses in the *flu* mutant, suggesting that H_2O_2 either directly or indirectly antagonizes $^1O_2$-mediated signalling (Laloi *et al,* 2007). Similarly, pre-treatment of *Chlamydomonas reinhardtii* with low concentrations of the $^1O_2$-generating photosensitizer rose bengal induced a rapid and transient acclimation to subsequently more intense $^1O_2$-producing stress but, conversely, increased its sensitivity to paraquat (Ledford *et al,* 2007). Therefore, $^1O_2$-mediated signalling might interact with H_2O_2-derived signals, suggesting that cells not only discriminate between different ROS but also that their responses to a given ROS can be modified by the concomitant perception of a second ROS. This discovery might explain previous observations suggesting that H_2O_2 can protect plants from high light stress. It seems that sensing the relative quantities of different ROS help to fine-tune the response to a specific stress.

Signalling versus cytotoxicity

An extensive second-site mutant screen of *flu* plants was undertaken to identify suppressor mutants that, during the 8 h dark period, accumulate similar excess amounts of free Pchlide as the original *flu* line but, on re-illumination, suppress $^1O_2$-mediated growth inhibition and cell death of mature *flu* plants and/or the bleaching of *flu* seedlings. So far, we have focused our studies on one particular group of suppressor mutants, known as *executer* (*ex*). Allelism tests and mapping of the mutated genes revealed that they represent only a single nuclear locus, *EX1*, which encodes a plastid protein unrelated to known proteins (Wagner *et al,* 2004). Under non-permissive dark:light conditions the *ex1/flu* double mutant generates similar amounts of $^1O_2$ as the parental *flu* line, but behaves similarly to wild-type seedlings as they remain viable and do not bleach (Fig 1, L/D), whereas mature plants continue to grow. It seems that because of the *ex1* mutation, the *flu* plants have lost the ability to perceive the presence of $^1O_2$ in their chloroplasts and to activate $^1O_2$-mediated response programmes. These results show that bleaching of *flu* seedlings, growth inhibition and cell death of mature *flu* plants cannot be ascribed to photo-oxidative damage by $^1O_2$, but instead reflect the $^1O_2$-mediated activation of genetically controlled response programmes that require the activity of the *EX1* gene. Enhanced levels of $^1O_2$ within plastids of the *flu* mutant not only trigger marked phenotypic changes but also modulate nuclear gene expression. Indeed, as previously mentioned, within the first 30 min of the release of $^1O_2$ rapid changes in nuclear gene expression occur. Inactivation of EX1 attenuated the upregulation of $^1O_2$-responsive nuclear genes but did not fully eliminate these changes. A second, related nuclear-encoded protein, known as EX2, has been identified that is also implicated in the changes in signalling of $^1O_2$-dependent nuclear gene expression (Lee *et al,* 2007). On inactivation of EX2 in the *flu* mutant, additional $^1O_2$-responsive genes emerge and genes already upregulated in *flu* plants are either stimulated further or downregulated. The primary function of EX2 seems to be that of a modulator attenuating and controlling the activity of EX1. When both EXECUTER proteins are inactive in the *ex1/ex2/flu* triple mutant, most of the $^1O_2$-responsive gene transcripts are close to wild-type level (Lee *et al,* 2007).

Both EX1 and EX2 are plastid-localized proteins that associate with thylakoid membranes (Lee *et al,* 2007). Therefore, the sensing of $^1O_2$ seems to take place within the plastid, in close vicinity to its production sites within plastid membranes of the *Arabidopsis flu* mutant (Przybyla *et al,* 2008; op den Camp *et al,* 2003). In wild-type plants, $^1O_2$ can be produced in the reaction centre of PSII and in the antenna system (Krieger-Liszkay, 2005). Whether these different production sites will affect differentially the sensing and signalling of $^1O_2$ is not yet clear. Indeed, a diffusion distance significantly larger than what was previously thought has recently been reported for $^1O_2$ in rat nerve cells (Skovsen *et al,* 2005). This indicates that sensing of $^1O_2$ might be distant from the production site. In *Chlamydomonas* cells under high light-stress (3,500 $\mu mol\ m^{-2}\ s^{-1}$), a small fraction of $^1O_2$ produced from the PSII reaction centre has been reported to leave the chloroplast and activate the nuclear gene *GPXH* (Fischer

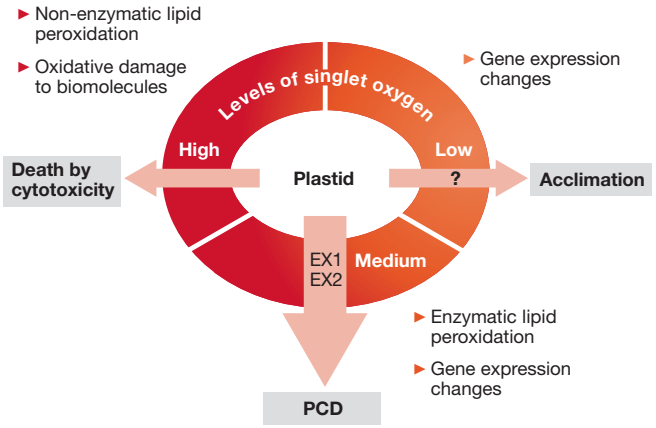

Fig 2 | Proposed model of plant responses to various levels of $^1O_2$. Constantly changing environmental factors can lead to the generation of variable levels of $^1O_2$ that can be mimicked in the *flu* mutant by modulating the length of exposure to darkness. Increasing dark incubation of *flu* plants leads to a proportional increase of free protochlorophyllide in the dark and of $^1O_2$ generation on illumination. When the production of $^1O_2$ is low, an acclimatory response might be activated that allows a plant to survive at subsequent higher levels of $^1O_2$. Greater amounts of $^1O_2$, for example in *flu* plants treated with 8 h of darkness and re-illuminated, trigger genetically controlled cell death through EXECUTER-dependent pathways. Most enzymatically oxidized lipid derivatives are produced under these conditions. When the level of $^1O_2$ increases further, plants die owing to chemical damage caused by $^1O_2$ and a massive production of non-enzymatically oxidized lipid is observed. EX, EXECUTER protein; PCD, programmed cell death.

et al, 2007). Whether EXECUTER proteins of *Chlamydomonas* are involved in mediating the $^1O_2$ effect has not yet been addressed.

In our initial experiments, we compared *flu* and *ex1/flu* mutants treated with single or repetitive dark periods of 8 h at the rosette leaf or seedling stages. Under these conditions, as described above, introduction of the *ex1* mutation suppresses growth inhibition and seedling lethality of the *flu* mutation. However, etiolated *ex1/flu* seedlings, which accumulate four- to fivefold the amounts of free Pchlide in *flu* and *ex1/flu* seedlings grown in continuous light and transferred to the dark for 8 h, bleach and die in a manner similar to the *flu* single mutant seedlings when transferred to light (Przybyla *et al,* 2008; Fig 1, D→L). This indicates that the amount of $^1O_2$ produced in *flu* plants after an 8 h dark:light shift does not reach a cytotoxic level and induces genetically controlled stress programmes, whereas etiolated *flu* seedlings transferred to light generate cytotoxic amounts of $^1O_2$ (Fig 2). In agreement with this idea, measurements of oxidation products of polyunsaturated fatty acids show that *flu* plants re-illuminated after being kept in the dark for 8 h accumulate enzymatically produced oxylipins, such as 13-hydroxy octadecatrienoic acid (13-HOT), 13-hydroxy octadecadienoic acid (13-HOD), oxophytodienoic acid and jasmonic acid, but not the products of non-enzymatic lipid peroxidation, such as 10-HOD, 12-HOD, 10-HOT and 15-HOT (Przybyla *et al,* 2008). By contrast, etiolated *flu* seedlings moved into the light accumulate large amounts of the non-enzymatically formed oxylipins 10-HOD, 12-HOD, 10-HOT and 15-HOT, which indicates that, under these more harsh conditions, toxic effects of $^1O_2$ prevail (Fig 2; Przybyla *et al,* 2008).

Perspectives

An interesting remaining question is whether $^1O_2$ levels lower than those produced in *flu* mutants after 8 h of darkness induce qualitatively different changes in gene expression that might lead to acclimation (Fig 2). This question could be addressed by first pre-exposing *flu* mutants to short dark periods that give rise to low concentrations of $^1O_2$ during re-illumination and then applying a much harsher stress treatment. Recent studies of *Chlamydomonas* indicate that sub-lethal stress leads to a transient or moderate elevation of $^1O_2$, and results in the activation of a subset of $^1O_2$-responsive genes and protection against a subsequent more severe stress (Ledford *et al,* 2007). The identification of differentially affected genes under conditions that induce genetically controlled cell death or acclimation will be important for modifying genetic constraints that determine the adaptability of plants to environmental changes.

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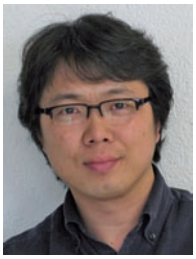

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